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Keratin 8 is a novel autoantigen of rheumatoid arthritis

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ABSTRACT

Objective: This research aims to verify Keratin 8 (K8) as a specific autoantigen in rheumatoid arthritis (RA).
Methods: First, total RNA was extracted from HaCaT cell to obtain cDNA by inverse transcription. Then, PCR was performed to amplify corresponding gene by K8 primers. Next, cloning, expression, and purification technology were used to obtain the recombinant human K8 (rhK8). At last, the purified rhK8, after identified by mass spectrometer, was used to perform further disease-related Western blotting and ELISA test with real clinical samples.
Results: Purified rhK8 was successfully obtained and then Western blotting confirmed antigenicity of K8 in rheumatoid arthritis. The reactivity of serum IgG against rhK8 was further detected in 34 of 50 RA patients (68%). The reactivity of RA serum IgG antibodies against K8 was significantly higher than healthy controls and systemic lupus erythematosus (SLE) patients.
Conclusion: This research confirmed Keratin 8 as a novel autoantigen of RA.

1. Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disease without a certain etiology. It typically manifests synovitis, multi-joint involvement and may lead to multisystem diseases such as atherosclerosis and lung diseases [1]. Sometimes, RA patients showed definitive chronic liver diseases, primary biliary cirrhosis [2,3], inflammatory bowel disease [4] and steatocystoma multiplex [5]. Since all these diseases were remarkably related to keratins [6], we considered keratins also had some uncertain connections with RA. Actually, there were already some antigens identified in RA, including heterogeneous nuclear ribonucleoproteins [7], enzyme peptidylarginine deiminase 4 [8], citrullinated peptide/protein antibodies, such as cyclic citrullinated peptide, citrullinated filaggrin and citrullinated vimentin, etc [9].

Keratin is the largest family of intermediate filament. Keratin had caused widespread concentration because of its wide distribution [10], multiple functions [11] and disease association. However, only a few reports talked about the roles of keratin in autoimmune diseases [6]. In 2013, keratin, type II cuticular Hb4 (KRT84/K84) was suggested as a specific antigen of RA with proteomic approaches [12]. Considering that K8 and K84 fall into similar sequence homology groups, type II [13] and K8 is also remarkably disease related [6], we assume that K8 may also be a autoantigen in RA. Actually, one of the useful auxiliary diagnoses was to detect the so called anti-keratin antibody (AKA) in clinical application, which was a useful marker for RA diagnosis as early as 1979 [14], but with the technology progress, the later so-called AKA's antigen was considered as filaggrin [15]. Afterwards, there were also antibodies, whose antigen might also be filaggrin, used in medical diagnosis called antibodies to human epidermis filaggrin (AFA) and anti-perinuclear factor (APF) [16,17]. However, AFA and APF's positivity of individuals was not identical to AKA's exactly, so there might be some special antigens exist in each antibody profile [18,19]. We also found protein sequences of K8 and filaggrin were not similar after comparison. We assumed that keratin 8 was another potential self-antigen of so called AKA.

The total prevalence rate of RA in China is 0.2–0.4% [20,21]. Although it is considering lower than worldwide prevalence rate (0.5–1%) [22], with China's large population, the number of RA patients is still very large, so more basic researches related to RA are needed. In this study, gene recombination technology was used to construct an expression system of K8 in *Escherichia coli*. Mass spectrometry was used to confirm purified rhK8 protein. The connection between K8 and patients was reassured by Western blotting and ELISA.

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2. Materials and methods

2.1. Sera collection

In this research, 167 Han Chinese subjects were enrolled and used to assess the serological criteria. Experimental group: 60 confirmed RA patients with an average of 52 years old (Range from 22 to 77, 43 female and 17 male patients). Disease controls: 50 SLE patients with an average of 33 years old (Range from 16 to 62, 41 female and 9 male patients). Healthy controls: 57 healthy people with an average of 25 years old (Range from 21 to 32, 47 female and 10 male). All 60 diagnosed RA patients met the criteria of the American College of Rheumatology [23]. Sera were stored at −80 °C immediately after aliquoted. All simples were provided by Chinese PLA General Hospital with ratification and all patients gave informed consents.

2.2. Cell line

The HaCaT cell line was provided by Cell Lines Service (Eppelheim, Germany). The condition of cultivation was set, according to our former research [24]. DMEM (HyClone, UT), containing 10% fetal bovine serum (HyClone, UT) was used to cultivate HaCaT. Then the cell was used to extract total RNA for next experiment.

2.3. Protein expression and purification

Protocols of gene recombination, protein expression and purification, certified by our previous study, were used [25]. In brief, total mRNA was extracted from aforementioned cell line by TRIzol reagent (Invitrogen, CA). The designed primer: Forward: (*Eco*R I) 5′-CCGGAATTCGTGACCCAGAAGTCCTACAAG-3′, Reserve: (*SaI* I) 5′-CGCGTCGACTTATTAATCAGAGGACTCAGACACCAG-3′, were used to amplify K8 gene by RT-PCR, carried out with the kit's instruction (Fermentas, MD). Then, the K8 gene was linked with pET-28(a) vectors, which were transformed into *E. coli* DH5α. Transformational *E. coli* DH5α strain was recovered in 400 μL Super Optimal Broth (SOC) cultivation for 1.5 h. Later, we transferred the bacterium culture solution to Luria Broth (LB) cultivation, containing kanamycin (50 μL/mL).

Extracting recombinant plasmids from *E. coli* DH5α and transforming these plasmids into high expression engineering strain *E. coli* BL21, using the same method that mentioned in this section. Under the optimal condition: 37 °C, 200 rpm shaking speed, overexpression of K8 was achieved by adding IPTG (1 mM) when optical density (OD) reaches 0.4–0.6. Finally, the recombinant K8 with N-terminal hexahistidine-tag was purified by Ni-NTA resin (Qiagen, Hilden, Germany), according to instruction supplied by manufacturer. After determining the concentration of protein by a BCA assay kit (Biosynthesis Biotechnology, Beijing, China), the purified protein was stored at −80 °C for further experiments.

2.4. Mass spectrometry

The method in our previous study was applied to this experiment [24,26]. Briefly, the target protein band was cut off from SDS-PAGE gel. After destaining the target gel pieces with 25 mM NH_4HCO_3 and 50% acetonitrile, the mixture was dried by a vacuum centrifuge. Then, 25 mM NH_4HCO_3 and 10 mM dithiothreitol were added to cover the target gel pieces and reduced for 2 h at 37 °C. The DTT solution was replaced by roughly same volume of 55 mM iodoacetamide in 25 mM NH_4HCO_3 and incubated for 45 min at room temperature in the dark. Next, 50% acetonitrile in 25 mM NH_4HCO_3 were used to wash the target gel pieces for 10 min. The liquid mixture was completely dried by vacuum concentrator. The dried target gel pieces were then digested by trypsin (Sigma, MO), mixed with 20 mL of 0.05 M NH_4HCO_3, overnight at 37 °C. The target protein band was identified, using a 4700 Proteomics Analyzer MALDI-TOF/TOF mass spectrometer (Applied Biosystems, Foster City, CA). Mass spectrometry data were analyzed with Mascot bioinformatics database search engine (Matrix Sciences, London, UK).

2.5. Western blotting

We used methods which were described in detail previously [24,27] with a few modifications. The purified rhK8 was loaded into 10% polyacrylamide gel and separated. Then, the target proteins were transfer to polyvinylidene fluoride (PVDF; Merck Millipore, MA) membranes. The PVDF membranes were blocked with 5% nonfat milk in PBS at 4 °C overnight. After blocking, we cut up PVDF membranes into strips and incubated them with sera of RA patients, disease controls or healthy controls (1:1000, diluted in 1% nonfat milk) overnight at 4 °C. Then, the PVDF membranes were washed by 0.2% PBST for three times to remove uncombined antibodies and each wash lasted for 10 min. Later, we Incubated the PVDF membranes with horseradish-peroxidase conjugated goat anti-human IgG (1:10,000, diluted in 1% nonfat milk) (ImmunoHunt, Beijing, China) for 1 h at 37 °C. The potential target bands were detected by enhanced chemiluminescence kit (Applygen, China) and exposed later.

2.6. Dot-ELISA

To perform this experiment, we referred to our previous research [28]. We spotted about 0.07 μg keratin 8 on the dull side of a 0.22-mm nitrocellulose membrane. After drying the membrane, 10% goat sera (diluted in PBS) was used to block the antigen discs for 1 h at 37 °C. Then the sera of RA patients (1:500, diluted in PBS) or healthy controls (1:500, diluted in PBS) were added to the discs and incubated for 1 h at 37 °C. PBST (phosphate buffered saline with 0.1% Tween-20, pH 7.4) was used to wash the discs for 3 times later. After removing the liquid phase and dry, the discs were incubated with goat anti-human IgG/HRP (1:1000, diluted in PBS) (ImmunoHunt, Beijing, China) for 1 h at 37 °C. Next, the washing process was repeated as aforementioned and 3, 3′-diaminobenzidine solution (ZSGB-BIO, Beijing, China) was added and colorated for 5 min. At last, adding deionized water to stop the reaction.

2.7. ELISA

The detection of potential autoantigen with ELISA was performed, according to previous studies [25,27]. Coating the 96-well microtiter plates with rhK8 (100 ng/mL, dissolved in 0.05 M carbonic buffer, pH = 9.6) overnight at 4 °C. After removing the liquid phase, each well was blocked with 100 μL goat sera (1:10, diluted in PBS) for 2 h at 37 °C. Then the liquid phase was removed again and the sera of RA, SLE patients and healthy controls (1:100, diluted in PBS) were used to incubate the 96-well microtiter plates for 2 h at 37 °C. Five-time wash was performed here to remove unbind autoantibodies. Next, 100 μL goat anti-human IgG/HRP (1:20,000, diluted in PBS) (ImmunoHunt, Beijing, China) was added to each well and incubated for 1 h at 37 °C. Again, wash each well with 0.1% PBST for five times to elute the second antibodies. Orderly, adding 50 mL tetramethylbenzidine (TMB) A (0.1 M citric acid, 0.2 M NA_2HPO_4, 0.6 g hydroperite/L) and 50 mL TMB B (5 mM citric acid, 0.4 mM EDTA-NA_2, 0.2 g TMB/L) to each well. After keeping the plates at a dark, room-temperatured place for 15 min, the reaction was stopped by adding 50 mL stop buffer (2 M H_2SO_4). Finally, OD value of each well was measured by ELISA reader (Tecan, Switzerland) at 450/620 nm.